

Identification of IEX-1 as a Biomechanically Controlled Nuclear Factor- κ B Target Gene That Inhibits Cardiomyocyte Hypertrophy

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Abstract—Biomechanical strain is a stimulus for cardiomyocyte hypertrophy and heart failure, but the underlying molecular mechanisms remain incompletely understood. Using an *in vivo* murine model of pressure overload and an *in vitro* model of mechanical stimulation of primary cardiomyocytes, we identified *iex-1* as a gene activated during the early response of cardiomyocytes to hypertrophic stimuli and as a gene product that inhibits hypertrophy without affecting cardiomyocyte viability. On stimulation of cardiomyocytes, *iex-1* mRNA and protein expression increased and translocation of the gene product to the cardiomyocyte nucleus occurred. *iex-1* has previously been proposed as a mediator of NF- κ B-dependent cell survival and growth in tumor cells. Here, we demonstrate that the biomechanical induction of *iex-1* in cardiomyocytes was NF- κ B-dependent, as overexpression of the NF- κ B inhibitor I κ B α completely inhibited strain-mediated *iex-1* mRNA accumulation. The functional role of *iex-1* was investigated by overexpressing wild-type *iex-1* with replication-defective adenoviral gene transfer. Overexpression of *iex-1* abolished cardiomyocyte hypertrophy by mechanical strain, phenylephrine, or endothelin-1 at levels that did not affect cell viability. These studies identify *iex-1* as a biomechanical stress-inducible and NF- κ B-dependent gene in cardiac muscle cells during the acute phase of hypertrophy with negative growth/regulatory effects that may counterbalance early hypertrophic responses in activated cardiomyocytes. (*Circ Res*. 2002;90:690–696.)

Key Words: hypertrophy ■ apoptosis ■ biomechanical strain ■ cardiomyocytes ■ heart failure

Biomechanical stress is a common pathway in the development of cardiac hypertrophy and failure. Responding to variations in mechanical load is an essential function of cardiomyocytes, allowing the myocardium to respond to rapid fluctuations in ventricular wall stress as well as chronic elevations of load in disease states. For yet unidentified reasons, the end-stage of chronic biomechanical overload *in vivo* is cardiac dilation and pump failure.

Mechanical strain and other hypertrophic agonists induce signaling events such as calcium fluxes,¹ activation of stress response protein kinases,² activation of the calcium-dependent protein phosphatase calcineurin,³ and NF- κ B translocation.^{4,5} These pathways may promote adaptive hypertrophic growth. The activation of these pathways, however, may also regulate the onset of cell death, and cell death may participate in the transition from myocardial hypertrophy to failure.⁶ Hirota et al.,⁷ for example, found that in mice with a deficient gp130-dependent myocyte survival pathway, biomechanical overload triggers cardiomyocyte apoptosis and early onset heart failure. The detailed molecular biology and the downstream genes activated by these signaling pathways, however, are incompletely understood.

In a previous study,⁴ we showed that biomechanical strain activates NF- κ B in cultured cardiomyocytes. Using the same model and DNA microarray technology, we identified the gene *iex-1* as a reproducibly mechanically induced gene in cardiomyocytes. *iex-1* is an immediate-early gene that participates in NF- κ B-dependent cell survival and growth,^{8–10} but whose expression and function in the heart has not been studied. In this study, we describe the activation of *iex-1* in cardiomyocytes stimulated to undergo hypertrophy by cyclic mechanical strain or by hypertrophic agonists *in vitro* and in acutely pressure-overloaded hearts *in vivo*. *iex-1* translocates to the nucleus on activation and prevents cardiomyocyte hypertrophy without inducing cell death when overexpressed. Thus, *iex-1* is a novel biomechanically responsive gene that may counterbalance early growth responses of activated cardiomyocytes.

Materials and Methods

Culture and Biomechanical Strain of Myocytes

Neonatal rat cardiac myocytes (NRCMs) from 1-day-old Harlan Sprague-Dawley rats (Charles River, Boston, Mass) were isolated by

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previously described methods.⁴ Mechanical deformation was applied to a thin and transparent membrane on which cells were cultured, an approach that produces controlled biaxially uniform cellular strain as well as visualization of cells.¹¹ After 24 hours of plating, NRCMs were washed twice with phosphate-buffered saline and then incubated with Dulbecco's modified essential medium (DMEM) containing 1% ITS supplement (Sigma) for 48 hours. To eliminate the variable of time-dependent changes due to cell age or effects of adhesion in each experiment, all cells were cultured on the membrane for an identical time period, and cells and media from all samples were harvested at the same time. For example, in a time course experiment with strain, the time point represents the time prior to harvest that strain was initiated, such that the strain sample and control sample were harvested at the same time.

Animal Studies

The Harvard Medical School Standing Committee on Animal Research approved the study protocol. Pressure overload was produced by transverse (chronic overload) or ascending (acute overload) aortic constriction. Male FVB mice (age 8 to 10 weeks) were anesthetized with pentobarbital (25 to 30 µg/g intraperitoneally) and the aorta was constricted by tying a 7-0 nylon suture around the vessel against a blunted 27-gauge needle with the aid of a dissecting microscope. Sham-operated animals were treated identically with the exception that the nylon suture was not tied. The needle was removed and hearts were harvested at the indicated time points. For the long-term studies, hypertrophy was measured by weekly echocardiograms. In the mice used for this study, mean left ventricular mass/body weight in banded mice compared with sham mice increased by 40% at 4 weeks and 64% at 12 weeks; these changes are comparable to increases measured in a large cohort of mice in our laboratory followed prospectively with blinded echocardiograms (data not shown).

Northern and Western Analyses

Northern analysis and Western analysis were performed as previously described.⁴ The primer set for the synthesis of the 502 base pair probe used to detect the transcripts of *iex-1* contained the 5'-TAACCACCTCCACACCATGA-3' and 5'-GTTCAAAGGCTCGAGGACAG-3' oligonucleotides and for *egr-1* the primer set contained the 5'-GTCAGTGGC-CTCGTGAGCAT-3' and 5'-AGGTGGTCACTACG-ACTGAA-3' oligonucleotides. Western analysis was performed using an affinity-purified rabbit polyclonal antiserum raised against a synthetic mouse/rat IEX-1⁽⁶⁷⁻⁸⁵⁾ peptide.

Recombinant Adenoviral Construction

The recombinant $\text{I}\kappa\text{B}\alpha$ adenovirus ($\text{I}\kappa\text{B}\alpha$ -Ad) expressing the porcine $\text{I}\kappa\text{B}\alpha$ gene and the recombinant β -galactosidase adenovirus (β -gal-Ad) were gifts of Dr Josef Anrather (Beth Israel Deaconess Medical Center; Boston, Mass). The recombinant control green fluorescent protein adenovirus (GFP-Ad) and recombinant adenovirus for *iex-1* (IEX-Ad) were generated with the Ad-Easy system and the pAdTrack-CMV vector as described previously.¹² Full-length *iex-1* was generated by PCR with the primers set containing the 5'-GAAGATCTACGTCTAAATTATGTGCCAC-3' and 5'-GCTTAGACCAGTTGGGATACCTTC-3' oligonucleotides containing *Bgl*II and *Xba*I restriction sites, respectively. cDNAs were cloned in the GFP-containing PadTrack-CMV shuttle vector (kindly provided by Dr Bert Vogelstein, John Hopkins Oncology Center, Baltimore, Md) and selected with kanamycin. After cloning, sequences were verified by DNA sequencing. Subsequently, clones were linearized, cotransformed with the adenoviral plasmid PadEasy-1 in electrocompetent BJ5183 cells, and selected with ampicillin. Recombinant plasmids were linearized and propagated in 293 cells. Viral titer was determined by GFP visualization. Stock titers were 10^9 pfu/mL for each vector.

Viral Gene Transfer

Cardiomyocytes were plated in 7% FCS overnight, incubated in DMEM containing 1% insulin, transferrin, selenium media supple-

ment (ITS; Sigma Chemical Co) for 24 hours and then incubated with adenovirus vector at a multiplicity of infection (MOI) of 20 in DMEM containing 1% ITS supplement. After an overnight incubation, the virus was removed and cells were incubated in DMEM containing 1% ITS supplement for an additional 24 hours. At this MOI, >99% of all cardiomyocytes were transduced, as determined by GFP expression or X-gal staining.

Immunocytochemistry

For immunocytochemistry, NRCMs were plated on silicone membranes or culture slides and exposed to strain, endothelin-1, or phenylephrine. After stimulation, cells were washed 3 times with phosphate buffer saline and fixed in 4% paraformaldehyde at 4°C overnight. Cells were then permeabilized with 0.1% Triton-X in phosphate buffered solution (PBS-T) for 10 minutes at room temperature. After 2 more washes, a blocking solution containing 10% rabbit serum in PBS-T was added. After 30 minutes, the cells were incubated with affinity-purified rabbit anti-IEX-1 antibody (1:200 in PBS-T) for 1 hour at room temperature then left overnight at 4°C. The secondary antibody (biotinylated rabbit anti-goat IgG, Vector Laboratories Inc, Burlingame, Calif) was diluted 1:200 in PBS-T. The incubation was carried out at room temperature for 1 hour. After 3 washes, cells were incubated for 1 hour at room temperature in avidin and then developed with a peroxidase DAB kit (Vector Laboratories Inc). No signal was detected when the first antibody was omitted or when preimmune rabbit immunoglobulins were used. To localize nuclei of the cells, a nuclear counter stain (hematoxylin) was applied to the sections.

Assays of Hypertrophy

Protein Synthesis

Cells were subjected to mechanical strain for 1 hour and then incubated in fresh DMEM containing ITS with 1.0 µCi/mL [³H]leucine for an additional 24 hours. The medium was aspirated, and the cells were washed twice with ice-cold PBS and once with 10% trichloroacetic acid (TCA; Sigma) and fixed for 45 minutes at 4°C with 10% TCA. After 2 washings with cold 95% ethanol, the radioactivity incorporated into the TCA-precipitable material was determined by liquid scintillation counting after solubilization in 0.1N NaOH. In addition, an aliquot was taken for determination of total protein.

Cardiomyocyte Size

Cells transfected with GFP-Ad or IEX-Ad were subjected to mechanical strain (4%, 1 Hz) or exposed to endothelin-1 (10 nmol/L) for 72 hours and then fixed in 4% paraformaldehyde. After mounting on coverslips, cells were visualized using an Olympus fluorescence microscope, and the surface area of the cardiomyocytes was calculated with image analysis software (Optimas 6.2). Cells from randomly selected fields in 3 independent cultured plates were examined (60 to 84 cells/group), and the surface area of cells from each group was determined and compared with control (ie, GFP-Ad, no strain).

Cell Death Assays

For the detection of cell death, 2 different assays were used. First, using flow cytometry, cellular DNA content was assessed as described previously.¹³ The percentage of cells with a sub-G1 DNA content was determined as a measure of apoptotic rate of the cell population. Second, TUNEL staining was performed with the *in situ* Cell Death Detection Kit (Boehringer Mannheim) according to the manufacturer's recommendations. To quantify the number of apoptotic cells, nuclei were counterstained with DAPI, and the total numbers of nuclei and TUNEL-positive nuclei were counted in 10 low-power fields in 2 independent experiments. More than 1500 DAPI-positive nuclei per conditions were counted in each condition.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously.⁴ Briefly, nuclear extracts were prepared and

incubated with [γ -³²P]dATP-labeled oligonucleotide probes for the NF- κ B response element consensus sequences (Promega). DNA-protein complexes were resolved with 6% nondenaturing polyacrylamide gel electrophoresis. Specificity was determined by supershift with anti-p50 antibody (15 μ g IgG/mL; Santa Cruz Biotechnology Inc) or excess unlabeled (cold) NF- κ B oligonucleotide to the nuclear extracts for 10 minutes before addition of radiolabeled probe.

Statistics

Each experiment shown was performed a minimum of 3 times. Data are presented as mean \pm SEM and were analyzed by 1-way ANOVA or Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

Results

Biomechanical Activation of *iex-1* in Cultured Cardiomyocytes

Cyclic biomechanical strain (8%, 1 Hz) induced *iex-1* mRNA accumulation in a time-dependent manner in primary cardiac myocytes, reaching a maximum between 1 and 6 hours (2.6 \pm 0.2-fold, $P < 0.05$, Figure 1A). In addition, when cardiomyocytes were subjected to cyclic biaxial strains of 0%, 4%, 9%, and 14% at 1 Hz for 4 hours, the induction of *iex-1* mRNA expression in cardiomyocytes was amplitude-dependent (Figure 1B).

We next investigated whether the increase in *iex-1* mRNA by mechanical strain was accompanied by changes at the protein level. Figure 1C shows a representative Western blot with the affinity-purified anti-IEX-1 antiserum that detects a single band around 22 kDa and illustrates that *iex-1* protein increased after 8 and 24 hours of strain (1.7 \pm 0.2-fold, $P < 0.05$). Mechanical strain also induced nuclear translocation of *iex-1*. Figure 2 shows cardiomyocytes, exposed or not exposed to strain for 2 hours (7%, 1 Hz), and stained with the affinity-purified anti-IEX-1 antibody. In cardiomyocytes not exposed to strain, *iex-1* was detected in the cytoplasm; 2 hours after strain a marked nuclear translocation was observed. Of note, in a sample of over 500 immunopositive cells, 54% of the immuno-positive cells in control conditions showed cytoplasmic localization, compared with only 1% to 3% in stimulated cells.

Biomechanical Activation of *iex-1* in the Intact Mouse Heart In Vivo

To determine whether *iex-1* is also mechanically regulated in vivo, we studied the effect of acute pressure overload on *iex-1* mRNA induction in mice with proximal aortic constriction. *iex-1* mRNA was rapidly induced (+2.2-fold, $n = 4$, Figure 3A) in hearts of pressure overloaded mice compared with those of sham-operated mice at 15 minutes, mimicking the rapid induction of *egr-1*, an early response gene exquisitely sensitive to ventricular pressure overload¹⁵ and used as positive control in these experiments. Induction of *iex-1* mRNA in vivo was accompanied by changes at the protein level (Figure 3B). Compared with sham operated mice, levels of left ventricular *iex-1* protein in banded mice (6 hours of constriction) increased 1.7 \pm 0.1-fold ($n = 2$).

Induction of *iex-1* mRNA by pressure overload was transient, diminishing to a 1.3-fold increase versus sham at 1 hour banding ($n = 3$) and a 1.1-fold increase versus sham at 3 hours

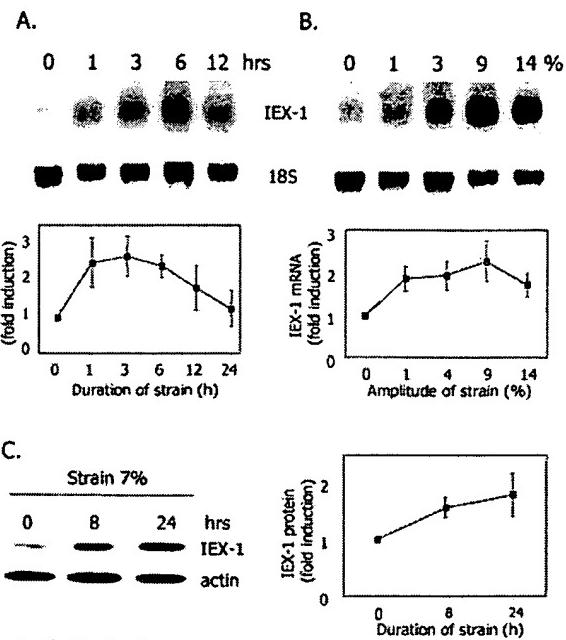


Figure 1. Biomechanical induction of *iex-1* in primary rat cardiomyocytes. A, Time-dependence of *iex-1* mRNA induction by mechanical strain in NRCMs. Myocytes were exposed to 0% or 8% cyclic mechanical strain (1 Hz). Northern analysis with ³²P-labeled *iex-1* cDNA probes. *iex-1* mRNA increased at 1 hour, peaked up to 6 hours, and returned to baseline after 24 hours. Graph with errors representing mean \pm SE (each time point represents the mean of 3 to 5 experiments). B, The amplitude-dependence of *iex-1* mRNA induction by mechanical strain in NRCMs. Myocytes were exposed for 4 hours to 0%, 1%, 4%, 9%, and 14% cyclic mechanical strain (1 Hz). Northern analysis with ³²P-labeled *iex-1* cDNA probes. Graph with errors representing mean \pm SE (each time point represents the mean of 2 experiments). C, NRCMs were exposed to 7% cyclic mechanical deformation (1 Hz) for 0, 8, or 24 hours. Cells were harvested and analyzed by Western analysis using the affinity-purified anti-IEX-1 (1:1000) antiserum or anti-actin antibody (1:1000), which served as a control for equal loading. The molecular mass of *iex-1* protein is 22 kDa. Graph with errors representing mean \pm SE (each time point represents the mean of 2 experiments).

banding ($n = 2$). This observation is consistent with the notion that *iex-1* is an immediate early gene. Furthermore, in long-term experiments, after 4 weeks or 12 weeks of aortic banding, levels of *iex-1* mRNA were not different from

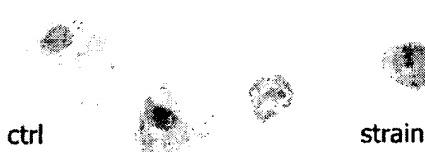


Figure 2. Nuclear translocation of *iex-1* in response to biomechanical strain. Immunocytochemical staining of cardiomyocytes showing nuclear translocation of *iex-1*. NRCMs were exposed to 7% cyclic mechanical deformation (strain, 1 Hz, 2 hours), fixed in 4% paraformaldehyde, and after permeabilization, stained with affinity-purified anti-IEX-1 (1:200) antiserum as described in Materials and Methods. To help localize nuclei of the cells, a nuclear counterstain (hematoxylin) was also applied.

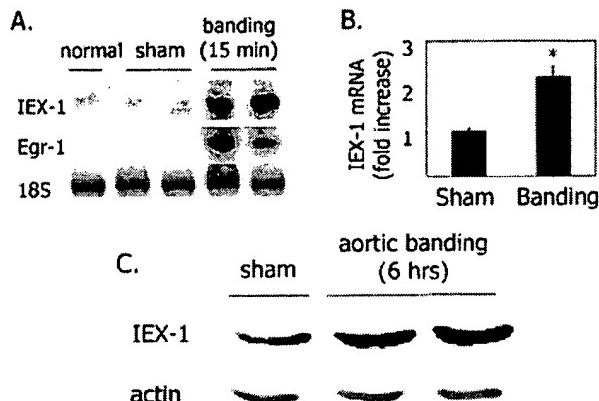


Figure 3. Induction of *iex-1* mRNA and protein by pressure overload in vivo. **A**, Male FVB mice were anesthetized with pentobarbital, and the ascending aorta was constricted as described in Materials and Methods. Hearts were harvested after 15 minutes, and mRNA from left ventricles prepared for Northern analysis. Northern analysis was performed with 32 P-labeled *iex-1*- or *egr-1* cDNA probes. Two separate duplicate experiments yielded similar results. **B**, Male FVB mice were anesthetized with pentobarbital and the ascending aorta was constricted as described in Materials and Methods. Hearts were harvested after 6 hours and analyzed by Western analysis using affinity-purified anti-IEX-1 antiserum (1:1000) or anti-actin antibody (1:1000), which served as a control for equal loading. The molecular mass of IEX-1 protein is 22 kDa.

sham-operated animals (data not shown), indicating that *iex-1* gene induction is an early and transient response to biomechanical stress, rather than a marker of chronic overload or hypertrophy.

Mechanisms of Biomechanical Induction of *iex-1* in Cultured Cardiomyocytes

iex-1 has been reported to be a target gene of NF- κ B,¹⁴ and we previously reported that mechanical strain induces NF- κ B-mediated transcriptional events in cardiomyocytes.⁴ To investigate the role of NF- κ B in *iex-1* mRNA accumulation, we overexpressed a specific inhibitor of NF- κ B activation,

I κ B α , by adenoviral gene transfer (I κ B-Ad). Ectopic expression of I κ B α abrogated mechanical induction of *iex-1* mRNA accumulation (Figure 4A) as well as IL-1 β -induced *iex-1* expression (not shown) when compared with *iex-1* induction in cells treated with the β -galactosidase control vector (β -Gal-Ad) (n=5). In Figures 4B and 4C, electrophoretic mobility shift assays with radiolabeled NF- κ B oligonucleotides are shown to illustrate that mechanical strain increased NF- κ B DNA binding activity. Experiments with IL-1 β served as a positive control for NF- κ B activation. Overexpression of I κ B α completely abrogated the activation of NF- κ B by mechanical strain and IL-1 β . The shifted complexes were specific for NF- κ B because they were supershifted in the presence of antibody to NF- κ B p65 subunit and disappeared with excess unlabeled oligonucleotide (Figure 4C).

Effect of *iex-1* on Cardiomyocyte Hypertrophy

To document the functional effects of *iex-1* in cardiomyocytes, replication-defective adenoviral vectors expressing wild-type *iex-1* were constructed. After demonstrating successful *iex-1* gene transfer (Figure 5A, insert), cardiomyocytes were infected with adenoviral vectors expressing GFP (GFP-Ad) or GFP and *iex-1* (IEX-Ad) at a MOI of 20, exposed to mechanical strain, and hypertrophic responses analyzed. At this level of infection, nearly 99% of cardiomyocytes were transduced, and a 3- to 5-fold increase in IEX-1 levels was obtained. Figure 5A shows that mechanical strain (1 Hz, 7%, 72 hours) significantly enlarged cardiomyocytes infected with the control GFP-Ad (1.41 ± 0.03 -fold versus control, $P < 0.05$). This response, however, was completely blunted in cardiomyocytes that overexpressed *iex-1* (1.04 ± 0.03 -fold). In addition, as shown in Figure 5B, mechanical strain (1 Hz, 7%, 24 hours) significantly increased rates of 3 H-leucine uptake in GFP-Ad/ β -Gal-Ad-infected cells (1.38 ± 0.05 -fold versus control, n=4, $P < 0.05$), but not in IEX-Ad/ β -Gal-Ad-infected cells (1.05 ± 0.04 -fold versus control, n=4, $P < 0.05$ versus unstrained control and versus strained GFP-Ad/ β -Gal-Ad), further suggesting that *iex-1* has a negative regulatory effect on hypertrophic growth. As

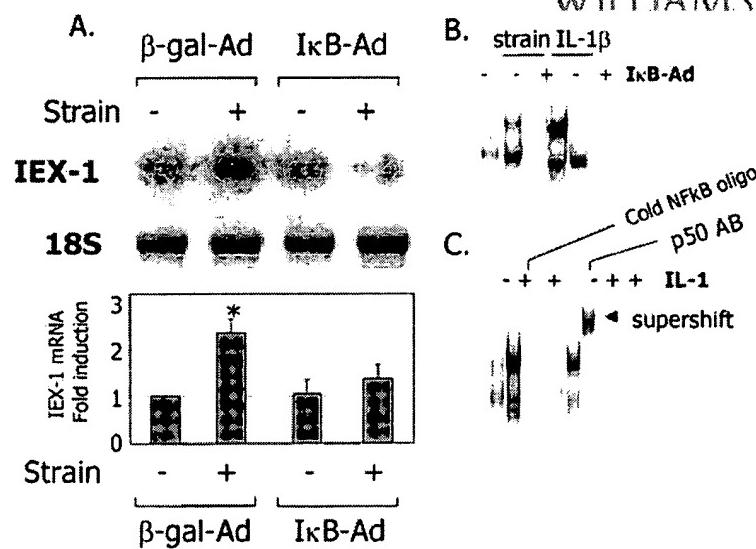


Figure 4. Role of NF- κ B in the mechanical regulation of *iex-1* expression. **A**, Effect of I κ B gene transfer on the induction of *iex-1* mRNA. Myocytes were infected with β -galactosidase adenovirus or with I κ B adenovirus and exposed for 4 hours to 8% cyclic mechanical strain (1 Hz). Northern analysis with 32 P-labeled *iex-1* cDNA probes. The upper panel shows a representative Northern analysis, the lower panel shows mean data of 5 experiments quantified by densitometry. * $P < 0.05$ vs unstimulated control. **B**, Electrophoretic mobility shift assay showing the effect of mechanical strain on NF- κ B DNA binding activity in normal cardiomyocytes and in myocytes with I κ B overexpression (I κ B-Ad). Myocytes were exposed for 2 hours to 0% or 9% cyclic mechanical strain (1 Hz) or to IL-1 β (10 ng/mL, used as a positive control for NF- κ B activation), in presence or absence of the I κ B vector. **C**, Specificity of signals was determined by addition of p50 antibody (Ab) (supershift) or unlabeled (cold) oligonucleotides representing NF- κ B consensus response elements to the nuclear extracts. Two separate experiments yielded similar results.

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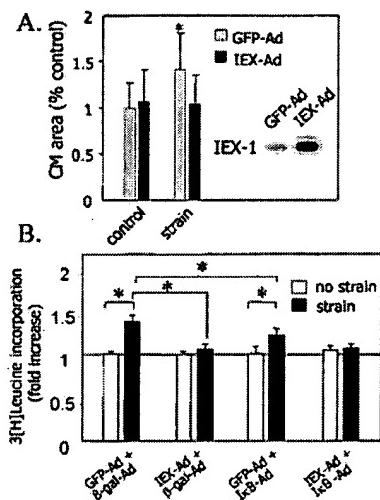


Figure 5. Influence of adenoviral-gene transfer of *iex-1* on cardiomyocyte hypertrophy. A, NRCMs were infected with GFP- or GFP/*iex-1*-expressing recombinant adenoviral vectors (GFP-Ad and IEX-Ad, respectively) and exposed to no strain (control) or to 7% cyclic mechanical deformation (strain) for 72 hours. Bar graphs with error bars represent mean \pm SD ($n=60$ to 85 cells). * $P<0.05$ vs unstimulated control. Insert, Expression of *iex-1* in primary rat myocytes in control conditions infected with GFP-Ad or IEX-Ad at a MOI of 20 and harvested 48 hours after infection for Northern analysis. B, NRCMs were coinjected with GFP-, β -gal-, GFP/*iex-1*-, or $\text{I}\kappa\text{B}\alpha$ -expressing recombinant adenoviral vectors (GFP-Ad, β -gal Ad, IEX-Ad, and $\text{I}\kappa\text{B}\alpha$ -Ad, respectively) and exposed to 0% (white bars) or 7% strain for 24 hours (black bars) in the presence of [^3H]leucine. Protein synthesis results are expressed as relative cpm/dish standardized to mean cpm of control cells in each experiment. Bar graphs with errors representing mean \pm SE ($n=3$ triplicate experiments). * $P<0.05$ vs indicated control.

further shown in Figure 5B, mechanical strain increased [^3H]leucine uptake in cardiomyocytes infected with $\text{I}\kappa\text{B}\alpha$ -Ad/GFP-Ad (1.20 ± 0.06 -fold increase, $n=4$, $P<0.05$), but when IEX-Ad was coinjected with $\text{I}\kappa\text{B}\alpha$ -Ad, this response was completely blocked (1.04 ± 0.04 -fold increase, $n=4$). Importantly, the hypertrophic response in cardiomyocytes overexpressing $\text{I}\kappa\text{B}\alpha$ was smaller than the response observed in cardiomyocytes infected with the GFP-Ad/ β -Gal-Ad control viruses (Figure 5B, $P<0.05$). These data are consistent with a recent report⁵ suggesting a participation of NF- κ B-dependent genes in cardiomyocyte hypertrophy. Thus, NF- κ B-mediated induction of *iex-1* during mechanical activation of cardiomyocytes does not seem to be related to the prohypertrophic actions of NF- κ B.

Effect of Neurohumoral Hypertrophic Factors on *iex-1* Expression and Hypertrophy

The experimental results above suggest that *iex-1* is a gene controlled by mechanical forces in cardiomyocytes, and that the *iex-1* gene product is involved in hypertrophic responses after mechanical strain. We next investigated whether other physiological stimuli associated with cardiomyocyte hypertrophy or cardiac remodeling besides mechanical strain increased *iex-1* gene expression in cardiac myocytes. Serum-starved primary cardiomyocytes were treated for 3 and 16 hours with various pharmacological agents and inductions

were compared with strain. Phenylephrine (PE, 10 $\mu\text{mol/L}$), endothelin-1 (ET-1, 10 nmol/L), interleukin-1 β (IL-1 β , 10 ng/mL), and leukemia inhibitory factor (LIF 1000 U/mL) induced *iex-1* to the same magnitude as strain, whereas angiotensin II (100 ng/mL) and tumor necrosis factor- α (10 U/mL) did not increase *iex-1* mRNA expression (Figure 6A).

Phenylephrine (10 $\mu\text{mol/L}$, 2 hours) and endothelin-1 (10 nmol/L, 2 hours) also induced nuclear translocation of *iex-1* as measured by immunohistochemistry (Figure 6B). Translocation was observed 2 hours after treatment of serum-starved cardiomyocytes, with kinetics similar to that induced by mechanical strain.

As shown above, forced expression of *iex-1* by adenoviral gene transfer blocked the hypertrophic effects induced by mechanical strain. Figure 6C shows that adenoviral gene

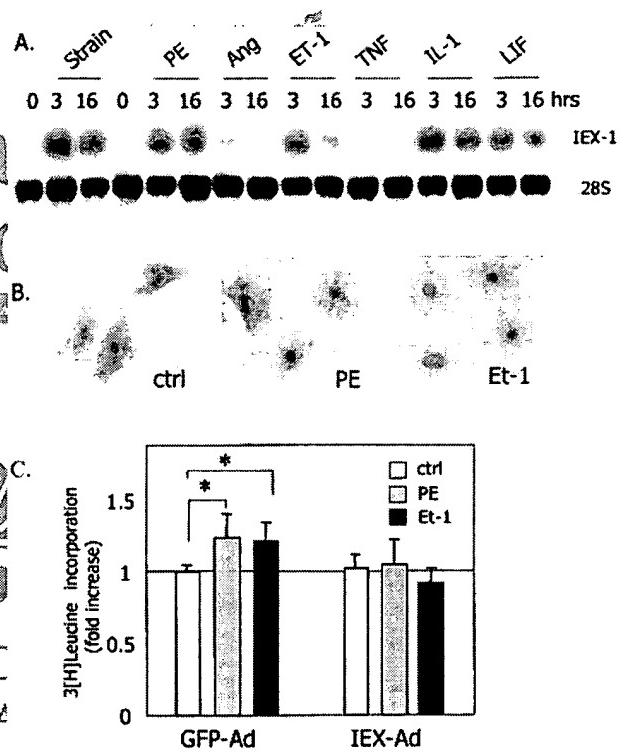


Figure 6. Effects of neurohormonal factors on *IEX-1* expression and hypertrophy. A, Myocytes were exposed to strain (7%) or treated with phenylephrine (PE, 10 $\mu\text{mol/L}$), angiotensin II (Ang II, 100 nmol/L), endothelin-1 (100 nmol/L), interleukin-1 β (IL-1 β , 10 ng/mL), tumor necrosis factor- α (TNF α , 10 ng/mL), or leukemia inhibitory factor (LIF, 1000 Units/mL) for 0, 3, or 16 hours. B, Immunocytochemical staining of cardiomyocytes showing nuclear translocation of *IEX-1*. NRCMs were exposed to phenylephrine (PE, 10 $\mu\text{mol/L}$, 2 hours) or endothelin-1 (ET-1, 10 nmol/L, 2 hours), fixed in 4% paraformaldehyde, and after permeabilization, stained with affinity-purified anti-*IEX-1* (1:200) antiserum as described in Materials and Methods. To help localize nuclei of the cells, a nuclear counterstain (hematoxylin) was also applied. C, NRCMs were infected with GFP- or GFP/*IEX-1*-expressing recombinant adenoviral vectors (GFP-Ad and IEX-Ad, respectively) and exposed to no stimulus (white bars) or to phenylephrine (10 $\mu\text{mol/L}$, gray bars) or to endothelin-1 (10 nmol/L, black bars) for 24 hours in the presence of [^3H]leucine. Protein synthesis results are expressed as relative cpm/dish standardized to mean cpm of control cells in each experiment. Bar graphs with errors representing mean \pm SE ($n=3$ triplicate or duplicate experiments). * $P<0.05$ vs indicated control.

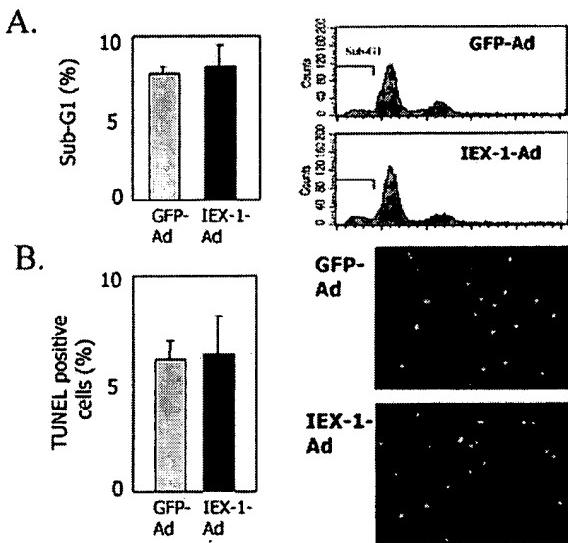


Figure 7. Influence of adenoviral-gene transfer of *iex-1* on cardiomyocyte viability. Myocytes were grown in serum-free conditions and infected with GFP- or GFP/*IEX-1*-expressing recombinant adenoviral vectors (GFP-Ad and IEX-Ad, respectively), 48 hours after infection. A, Cells were analyzed for DNA content by flow cytometry. The percentage of cells with a sub-G1 DNA content was taken as a measure of apoptotic rate of the cell population. Bar graphs with errors representing mean \pm SE ($n=4$). B, Apoptotic cells were identified by TUNEL staining. For quantification, nuclei were counterstained with DAPI (not shown), and the total numbers of nuclei and TUNEL-positive nuclei were counted in 10 low-power fields in 2 independent experiments. About 1500 nuclei were counted for each transduced sample. Bar graphs with errors representing mean \pm SE.

transfer of *iex-1* also inhibited hypertrophic responses by phenylephrine and endothelin-1. When cardiomyocytes were stimulated with phenylephrine (10 μ M, 24 hours) or endothelin-1 (10 nM/L), [3 H]leucine uptake increased significantly in GFP-Ad-infected cells (1.24 ± 0.17 - and 1.21 ± 0.13 -fold versus control, respectively, $n=3$, $P<0.05$), but not in IEX-Ad-infected cells (1.04 ± 0.18 - and 0.92 ± 0.10 -fold versus control, respectively).

Effect of *iex-1* on Cardiomyocyte Viability
We next examined whether overexpression of *iex-1* affected cell viability of cardiomyocytes. Cardiomyocytes were grown in serum-free conditions, infected with GFP-Ad or IEX-Ad, and harvested for flow cytometry or TUNEL staining. The incidence of apoptotic cell death (sub-G1 fractions or incidence of TUNEL-positive cells) was not different in GFP-Ad- and IEX-Ad-infected cells and comparable with measurements by previous investigators in similar culture conditions^{16,17} (Figure 7).

Discussion

The response of the heart to biomechanical overload depends on the activation of hypertrophic and survival pathways. In the present study, we identified the regulation of *iex-1*, a gene previously described as a mediator of NF- κ B-dependent growth and survival, during early hypertrophic responses in the murine pressure overloaded ventricle and in isolated

cardiomyocytes. We demonstrated that forced expression of *iex-1* may inhibit hypertrophy, does not induce cell death, and is a downstream effector of NF- κ B when cells are activated by mechanical strain.

iex-1 was previously described as an early response gene expressed during stimulated proliferation in fibroblasts¹⁸ and epithelial cell lines.¹⁹ In addition, *iex-1* was a member of a small set of induced genes in a genomic analysis that differentiated metastatic versus nonmetastatic melanoma cells.²⁰ The function of *iex-1*, however, is unclear. Some studies have previously suggested a role for *iex-1* in the control of cell viability, whereas others have focused on its effects on cell growth. Arlt and coworkers¹⁰ reported that overexpression of *iex-1* accelerated growth of HeLa cells and augmented susceptibility to apoptosis. Segev and coworkers⁸ described the regulation of *iex-1* in breast cancer cells and inhibiting growth of these cells when overexpressing *iex-1*. The biochemical pathways underlying these regulatory effects, however, are unclear.

Consistent with the latter report in breast cancer cells, our data suggest a role for *iex-1* in the growth of cardiomyocytes. In contrast with the cells used in previous reports, however, cardiomyocytes are terminally differentiated and generally respond to growth-promoting stimuli with hypertrophy rather than with hyperplasia. *iex-1* was induced by several hypertrophic stimuli including mechanical strain, phenylephrine, and endothelin-1. When overexpressed, *iex-1* completely abrogated hypertrophic responses to mechanical strain, phenylephrine, and endothelin-1. Importantly, this negative regulation of hypertrophy was not accompanied by cardiomyocyte apoptosis, suggesting that levels of *iex-1* producing no apparent deleterious effects in cardiomyocytes may interact specifically with components of the hypertrophic pathway.

Intriguingly, the inhibitory effects of *iex-1* on hypertrophy resemble the action of 2 other recently described hypertrophy inhibitory genes in cardiomyocytes, namely myocardium-enriched calcineurin inhibitory protein-1 (MCIP-1), an endogenous inhibitor of calcineurin,²¹ and suppressor of cytokine signaling-3 (SOCS-3),²² an intrinsic inhibitor of JAK. Similarly as *iex-1*, SOCS-3 is induced immediately after aortic constriction and shows a transient induction, but when its expression is forced through adenoviral gene transfer, SOCS-3 inhibits cardiomyocyte hypertrophy.²² Endogenous inhibitors of hypertrophy may cooperate in a negative feedback circuit that ensures the termination of the cardiac hypertrophy response. Immediate and transient activation of growth inhibitory switches on activation of cardiomyocytes may also counteract repetitive and transient growth stimuli such as physical exercise. Endogenous inhibitors of hypertrophy in general may help to diminish deleterious effects of unrestrained hypertrophy and may prevent, or at least delay, the transition to ventricular failure.

The molecular interactions of *iex-1* underlying its growth-regulating effects in cardiomyocytes or in tumor cells are still unknown and deserve further study. The marked translocation of *iex-1* protein on stimulation, however, suggests that its main action may be in the nucleus. Structurally, *iex-1* protein does not contain functional elements that reveal a role of *iex-1* as a transcriptional factor, but it will be interesting to study

whether *iex-1* directly or indirectly affects transcriptional growth events by interacting with other factors.

Induction of *iex-1* by mechanical strain was mediated by activation of the transcription factor NF- κ B. The presence of functional binding sites for NF- κ B in the *iex-1* promoter has been shown previously, but other regulatory components have been proposed as well.^{8,14} In cardiomyocytes, NF- κ B is activated by several hypertrophic stimuli including mechanical strain.^{4,5} The net effect of NF- κ B activation in cardiomyocytes seems to be induction of hypertrophy.⁵ Accordingly, NF- κ B-mediated induction of *iex-1* in cardiomyocytes seems to be opposite to the prohypertrophic actions of NF- κ B. In a striking parallelism with relation to apoptosis, *iex-1* was described as an NF- κ B-dependent cell apoptosis sensitizing gene, in studies where the overall activity of NF- κ B was death protection.¹⁰ Thus, *iex-1* may comprise part of the counterregulatory processes initiated by NF- κ B.

In conclusion, the present study identified *iex-1* as a growth inhibitory switch activated immediately and transiently after onset of cardiomyocyte hypertrophy *in vitro* and *in vivo*. This finding suggests that early negative feedback responses may temporarily control myocardial hypertrophic responses.

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